ISOLATION AND CHARACTERIZATION OF *IN VITRO* RADIOACTIVELY LABELLED SH-PROTEINS FROM RAT LIVER MITOCHONDRIAL MEMBRANES

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1. Introduction

There is a correlation between mitochondrial SH-groups and K^+ [1, 2] and phosphate [3–5] transport. Regulation of cation transport was reported to be connected with the oligomycin sensitive ATPase system [6]. An influence of maleimides on swelling and ATPase activity has been reported [7].

This paper aims at the localization of reactive sulfhydryl groups in the mitochondrial membrane proteins by the use of radioactive maleimides. Two independent isolation procedures were designed: First, the mitochondria were labelled by a small concentration of ^{35}S-*N*-(*N*-acetyl-4-sulfamoylphenyl)maleimide (ASPM) [8] during incubation in tris-KCl buffer pH 7.5. The mitochondrial membranes were isolated, subsequently solubilized and chromatographic and electrophoretic separations were performed. Second, the F_1 ATPase [9] was isolated from sonicated mitochondria, labelled *in vitro* with ^{14}C-NEM. It is shown that radioactivity is found in the basic structural proteins, and not significantly in the oligomycin insensitive ATPase protein.

2. Materials and methods

^{35}S-ASPM, specific activity 27 mCi/mmole at the date of delivery, was prepared by Farbwerke Hoechst A.G., Frankfurt. ^{14}C-NEM was purchased from Schwarz Bioresearch Inc., Orangeburg, New York, specific activity 10.3 mCi/mmole. NEM was obtained from Schuchardt, München, and purified by sublimation. Protamine sulfate was purchased from Serva Heidelberg, aquacide from Calbiochem, Luzern.

Wistar rats of a local strain, weighing 120–150 g were used. The mitochondria were prepared essentially by the conventional manner as described previously [10]. The final stock suspension was about 8–10 ml.

2.1. *Labelling with ^{35}S-ASPM*

The incubations were performed in KCl 0.125 M; tris 0.02 M, adjusted to pH 7.5 with HCl (tris-KCl buffer) containing 5 μM ASPM. The mitochondria were pipetted into this medium at 24° (protein concentrations about 0.18 mg/ml tris-KCl, determined by the method of Lowry [11] in sonicated mitochondria) and the reaction stopped by a 20 fold excess (calculated versus ASPM concentration) of cysteine after 15 sec. The suspensions were then centrifuged for 12 min at 25,000 *g* (5°). The pellets were washed superficially and resuspended in 0.25 M sucrose. Sonification for 90 min at 5° and 3.5 A and the subsequent differential centrifugation at 13,500 *g* and 100,000 *g* were accomplished in the usual way [10]. The mitochondrial membranes were then dissolved in 1% solutions of dodecylsulfate and digitonin each for about 6 hr. The dissolved membranes were lyophilized, and afterwards filtered through a Sephadex G-75 column (fig. 1). Peak 1 of several gel filtrations was pooled and concentrated in the air stream at 5° to 7–10 ml. The precipitated proteins were redissolved during reduction with $Na(BH_4)$ as described in [10]. After exhaustive dialysis against H_2O adjusted to pH 9 with NH_3, the proteins were concentrated to a volume of about 15 ml and chromatographed on a DEAE-Sephadex A-50 column (fig. 2). The whole radioactive peak was dialysed against H_2O/NH_3, pH 9, and cellulose acetate electrophoresis was performed (fig. 3).

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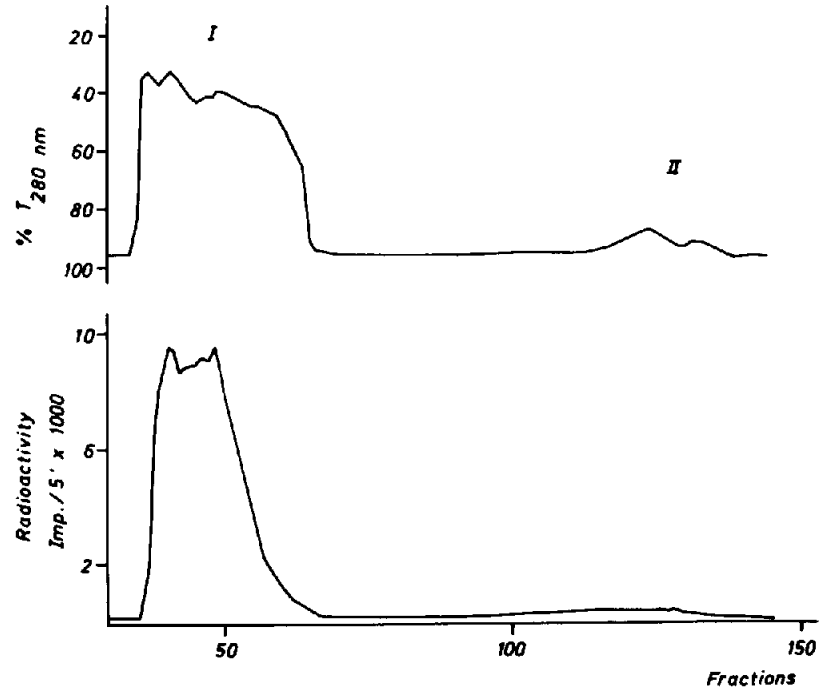

Fig. 1. Sephadex G-75 gel filtration. A column of 12 X 1400 mm was equilibrated with 0.01 M K_2HPO_4, 0.5 M NaCl, pH 8.6. About 450 mg of lyophilized mitochondrial membranes after dodecylsulfate and digitonin treatment were layered onto the column. % $T_{280\ nm}$ was measured continuously in an LKB Uvichord, radioactivity was determined in 0.2 ml of the fractions.

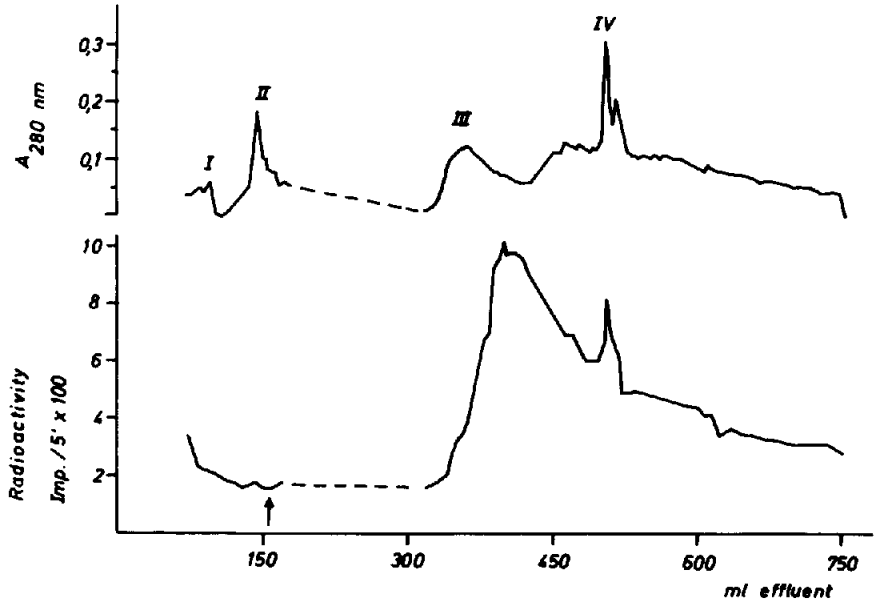

Fig. 2. DEAE-Sephadex A-50 chromatography. A column of 25 X 310 mm was equilibrated with 0.01 M tris-HCl, pH 8.5. 15 ml of the reduced proteins (50–60 mg protein) after Sephadex G-75 gel filtration were layered onto the column, and eluted for 24 hr with the equilibration buffer. Afterwards a gradient with 2 M NaCl (500 ml mixing flask) was started (arrow). Absorption at 280 nm was measured in a Zeiss photometer. Radioactivity was determined in 1 ml aliquots of the dialysed fractions.

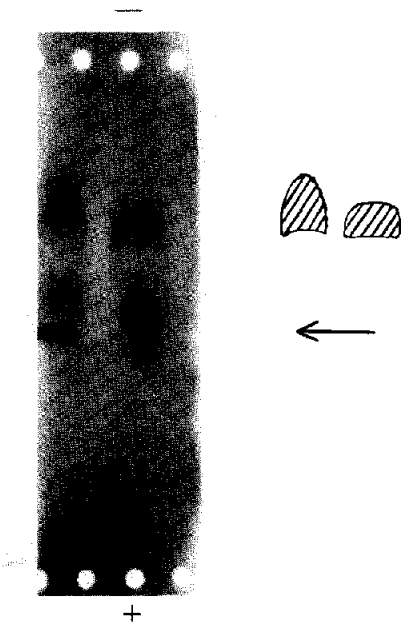

Fig. 3. Cellulose acetate electrophoresis of the radioactive peak after DEAE-Sephadex chromatography. About 0.25 mg of lyophilized protein was applied to a strip of cellulose acetate, and subjected to electrophoresis for 25 min at 2 mA.
Buffer system: 29.34 g sodium barbital, 19.42 g sodium acetate, 180 ml 1 N HCl, made up to 3000 ml with aqua bidest, pH 8.6, 6 M urea.
//// Radioactivity (autoradiographically); ← Origin; Staining: Coomassie blue.

2.2. *Labelling with ^{14}C-NEM*

The mitochondria were incubated in the tris-KCl buffer, pH 7.5, as described above, but with 0.5 μM ^{14}C-NEM. After standing for 30 sec at 24°, the suspension was centrifuged for 6 min at 20,000 *g* (5°), without cysteine. Immediately afterwards, the pellets were washed superficially with 0.25 M sucrose to remove the incubation medium and resuspended in 0.25 M sucrose, 0.01 M tris-SO_4, 2 mM EDTA pH 7.4 (sucrose-tris-EDTA); sonification was carried out in this medium for 30 min at 2.5–3 A [10]. The supernatant, after centrifugation at 17,500 *g* for 20 min, was made up to 102 ml with sucrose-tris-EDTA and allowed to stand over night at 24° (see table). The subsequent procedure was performed according to Penefsky [9]. After the addition of about 10–20 ml of 0.5% prot-

Table
Preparation of the mitochondrial ATPase (F_1) according to Penefsky [9], after incubation of the mitochondria *in vitro* with 0.5 μM ^{14}C-NEM.

Imp./5 min	in ml	ml total	mg protein	Step of fractionation
34,624	0.2	103	142	sonicated mitos.
32,494	0.2	102	54	S 17.5
28,503	0.2	92	50	S pH 5.4
28,735	0.2	89	49	S_1 PS
25,601	0.2	102	48.5	S_2 PS
189	0.2	3.5	0.7	R_2 PS
335	0.5	3.5	0.7	R_2 PS

Abbreviations:
S 17.5 : supernatant after 17,500 *g*
S pH 5.4: supernatant after adjustment to pH 5.4
S_1PS : supernatant after the first step of protamine sulfate precipitation
S_2 PS : supernatant after the second step of protamine sulfate precipitation
R_2 PS : precipitated protein after the third step of fractionation = ATPase protein.

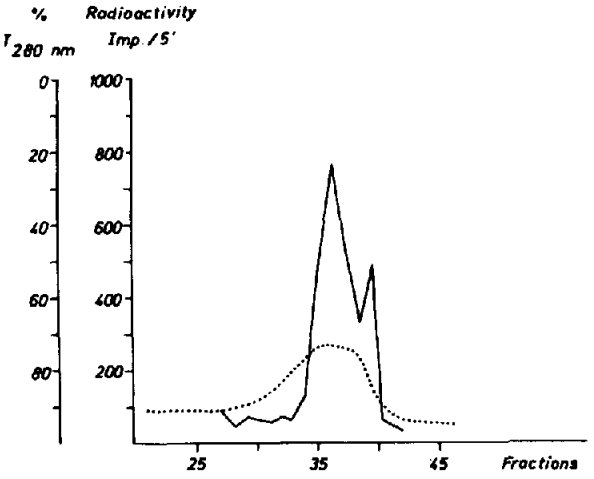

Fig. 4. Sephadex G-25 gel filtration of the supernatant after protamine sulfate precipitation. A column of 12 × 800 mm was equilibrated with 0.5 M NH_4HCO_3 adjusted to pH 8.7 with NH_3. About 1/3 of the concentrated supernatant after protamine sulfate precipitation (about 15 mg protein) was layered onto the column. Radioactivity was determined in 0.2 ml aliquots of the fractions.
..... % T_{280} nm;
——— Radioactivity.

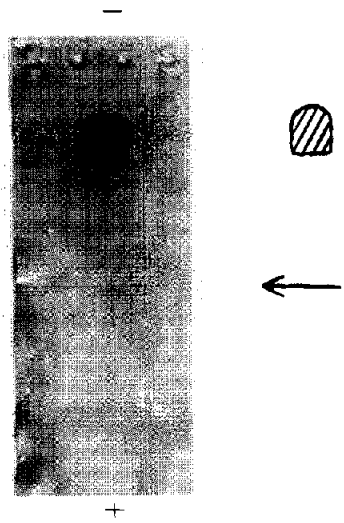

Fig. 5. Cellulose acetate electrophoresis of the lyophilized radioactive peak shown in fig. 4. The conditions were the same as in fig. 3.

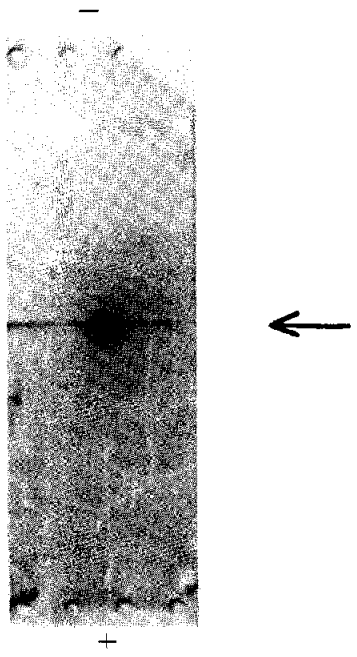

Fig. 6. Cellulose acetate electrophoresis of the ATPase protein (F_1). The precipitated protein (R_2 PS of the table) was electrophoresed after gel filtration (Sephadex G-25) and dialysis. About 70 μg of protein was applied to the strip of cellulose acetate. The other conditions were the same as in fig. 3.

amine sulfate, the ATPase protein precipitated and was centrifuged. ATPase activity was determined according to the previous communication [7]. The supernatant was concentrated and chromatographed on Sephadex G-25 (fig. 4). The radioactive fractions were subjected to a cellulose acetate electrophoresis (fig. 5). The ATPase protein, after the third step of the preparation [9] – gel filtration on Sephadex G-25 (column 12 × 800 mm, equilibrated with sucrose-tris-EDTA pH 7.4) and subsequent dialysis – was likewise electrophoresed on cellulose acetate (fig. 6).

3. Results and discussion

As shown in the figs. 3 and 5, radioactivity emerges in the highly basic fractions of the membrane proteins. It is evident from the table that the oligomycin insensitive ATPase (F_1) had no significant radioactivity. Mac Lennan and Tzagoloff [12], Mac Lennan and Senior [13] point out that structural proteins exhibit a band in the disc electrophoresis running parallel with the oligomycin sensitivity conferring protein (OSCP). It is postulated that the labelled SH-groups shown here belong to this protein species, as radioactivity is found in the protein which moved farthest towards the cathode. The oligomycin insensitive ATPase protein migrates mainly with the neutral, weakly anodic protein (fig. 6). Moreover, labelling with maleimide was found to be oligomycin sensitive (in preparation). It is concluded that effects of maleimides at low concentrations on the mitochondrial ATPase system are mediated through action on structural proteins.

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